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PRINCIPAL INVESTIGATOR: Lesia Dropulic, M.D.

CONTRACTING ORGANIZATION: The Johns Hopkins University School of Medicine
Baltimore, Maryland 21205-2196

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Lesia Dropulic, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Johns Hopkins University School of Medicine
Baltimore, Maryland 21205-2196

E-Mail: lesia@mail.jhmi.edu

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The purpose of the proposed research is to develop a propagation-competent alphavirus vector that is targeted specifically to receptors expressed on breast cancer cells or to receptors expressed on tumor-associated vasculature. We hypothesize that this type of targeted vector would provide a very efficient means of specifically killing a large number of malignant cells. Sindbis virus (SV), an alphavirus, containing a 200 basepair sequence encoding the epidermal-like growth factor domain of heregulin in place of a portion of its receptor-binding domain, is impaired in its ability to assemble and bud from transfected cells. However, a SV containing a 13 amino acid NGR-containing peptide motif is able to replicate and spread in infected cells. This NGR-containing SV exhibits an ability to more efficiently kill cells expressing the targeted CD13 receptor compared to parent virus-infected cells. Hence, we have demonstrated that it is possible to generate a propagation-competent SV containing a targeting ligand. The optimal location(s) for such a ligand within the receptor-binding domain of SV needs to be determined. Subsequently, in vivo studies using mouse tumor models will be performed to determine the ability of such a targeted SV to specifically destroy breast cancer tumors.

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I. Introduction. Two main challenges of cancer gene therapy are the development of vectors targeted specifically to tumor cells and the efficient delivery of the therapeutic agent to all or to the majority of tumor cells. Addressing these two issues, we intend to develop Sindbis virus (SV), an alphavirus, into a novel vector for breast cancer gene therapy. The advantages of SV vectors include lack of serious disease caused by SV in humans, the ability of SV to infect nondividing and dividing cells, no risk of insertional mutagenesis because SV is an RNA virus, and the ability to produce high titer stocks and achieve high level of heterologous gene expression. Since SV kills cells by apoptosis, specific destruction of tumor cells will occur if the virus is targeted to tumor cells (4). Furthermore, use of a propagation-competent viral vector will provide a very efficient means of obtaining access to most or all of the tumor cell population. As a first step, erbB-2, a tumor-associated growth factor receptor over-expressed in 20-30 % of human breast carcinomas will be used as a model target. The EGF-like domain of heregulin was cloned to replace portions of the receptor-binding domain of the E2 glycoprotein to target ErbB-2 overexpressing breast cancer cells. As an additional approach, an NGR-containing peptide motif that binds to the CD13 receptor on tumor associated vasculature was used to modify the receptor-binding domain of E2. The long-term goal of this proposal is to develop target-specific SV vectors for application to breast cancer therapy by modifying the SV E2 envelope glycoprotein with ligands that recognize specific cell surface receptors on breast cancer tumors.

II. Body.

Task 1 of statement of work: Construct targeted viral vectors.

As reported in the previous annual report, replacement of portions of the putative receptor-binding domain of the E2 glycoprotein with heregulin resulted in a significant attenuation of virus replication and in an inability to generate a working recombinant virus stock. The following experiments were performed to try to understand what stages of virus replication are affected by the presence of the EGF-like domain of heregulin within the E2 glycoprotein:

1) Immunoflurescence assay to look for expression of heregulin on the surface of cells (please refer to manuscript in preparation, figure 1, for schematic diagram of constructs. Figure 3 from the manuscript is reproduced below).

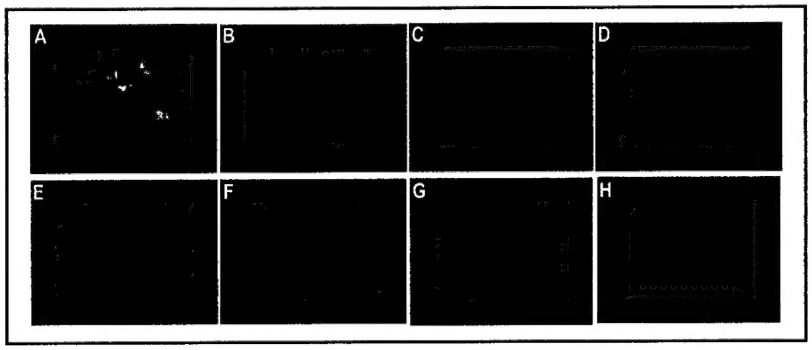

Figure 3 of manuscript. Immunofluorescence assay. BHK (A-D) and SKBR3 (E-H) cells were transfected with SVher.15 RNA (A,C,E,G) or yeast RNA (B,D,F,H), fixed with 4 % paraformaldehyde and stained with anti-SV rabbit serum (A,B,E,F) or with anti-heregulin antibody (C,D,G,H). Similar results were obtained with SVher.12 RNA. Parent virus RNA-transfected cells were fluorescent when stained with anti-SV rabbit serum, but not when stained with the anti-heregulin antibody (not shown).

These results indicated that the chimeric heregulin-E2 protein was transported to the surface of the cells where it could be incorporated into the envelope of the budding virions.

2) Radiolabelling and pelleting virus (figure 2 from manuscript). To determine whether the chimeric E2 was incorporated into Sindbis virions, recombinant viral RNA was transfected into BHK cells. The cells were radiolabelled and the virions were pelleted from supernatant fluids (figure 2C).

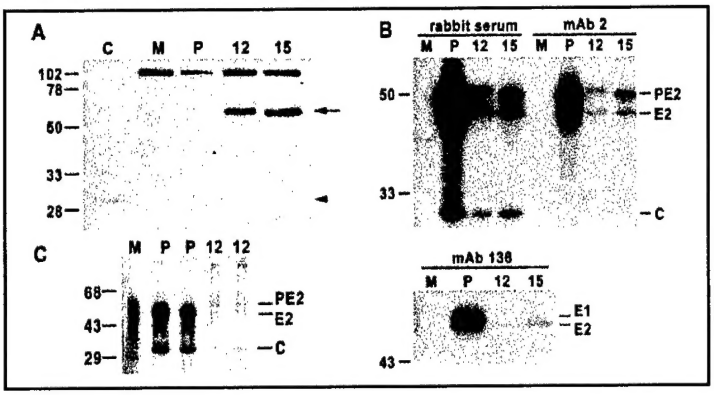

Figure 2 from manuscript. Panel **C.** Pelleted radiolabelled heregulin-containing virions. M, mock-transfected supernatant. P, parent virus RNA-transfected supernatant. 12, SVher.12-transfected supernatant.

A very small amount of virus was pelleted from the supernatant fluids, indicating that the heregulin sequence impaired virion assembly and release. The post-transfection supernatant fluids were used to infect BHK and SKBR3 cells and no cytopathic effect was observed. Also, titering the supernatant fluids on BHK cells resulted in no plaque formation. These data indicated that the heregulin sequence had a significant effect on virus release and possibly on virus binding and entry.

3) To try to isolate mutant heregulin-expressing viruses that are propagation competent, we have begun to perform transfection-overlay assays. Viral RNA was transfected into BHK cells and 4 hours after transfection the cells were overlayed with agarose. Two to 3 days after transfection, the monolayers were observed for plaque formation. So far, we have isolated one potential heregulin-containing mutant that we are in the process of characterizing.

Task 2 of statement of work: Test the targeted SV vectors for specificity of binding and specific infectivity of erbB-2-overexpressing breast cancer cells.

1) To determine whether the cytopathic effect (figure 4 of manuscript) and cell death (figure 5 in manuscript) seen in SKBR3 breast cancer cells after transfection with viral RNA was the result of a ligand-receptor interaction, competition assays in the presence of a GST-α-heregulin fusion protein (figure A) and an anti-erbB-3 receptor-blocking antibody (figure B) were performed. No difference in the amount of cell death was detected in the presence of GST-α-heregulin or of the anti-erbB-3 receptor-blocking antibody.

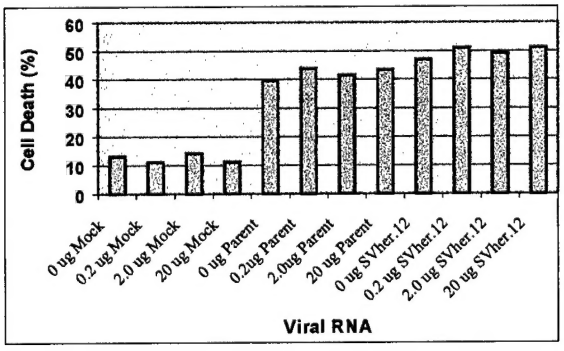

Figure A. Cell death in the presence or absence of GST-α-heregulin 48 hours after transfection of SKBR3 cells. Zero, 0.2, 2.0 or 20 μg of heregulin was added to the cultures 4 hours after transfection. The amount of cell death in the cultures was quantified via a propidium iodide (PI) assay using a flow cytometer.

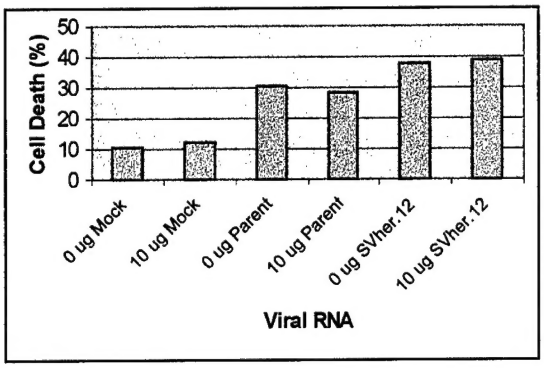

Figure B. Cell death in the presence or absence of an anti-erbB-3 receptor-blocking antibody (Labvision) 48 hours after transfection of viral RNA. The amount of cell death in the cultures was quantified via a PI assay and flow cytometry.

To determine whether a spreading SV infection is causing death of the breast cancer cells, similar experiments in the presence of anti-SV rabbit serum or in the presence of a neutralizing monoclonal antibody are underway.

2) To determine whether the ability of the heregulin-containing viral RNA to kill breast cancer cells was dependent on the level of expression of the erbB family of receptors, various breast cancer cell lines expressing different levels of erbB-2 and erbB-3 were transfected with SVher.12 or SVher.15 RNA (figure 5 of manuscript).

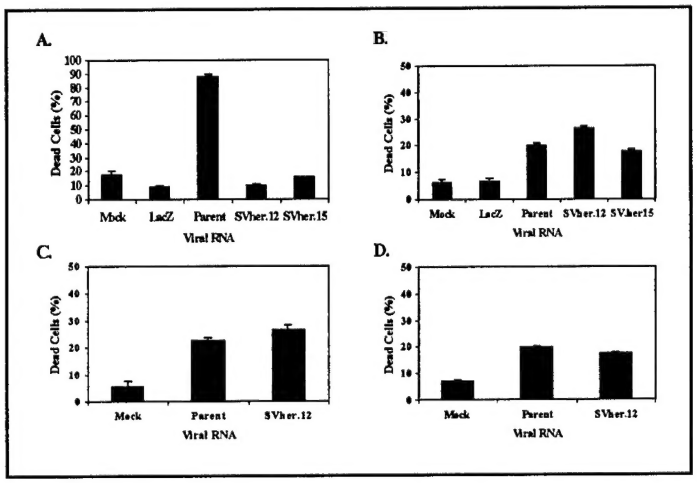

Figure 5 of manuscript. Quantification of cell death in transfected mammalian cell lines. Forty-eight hours after transfection, the cells were harvested and stained with Trypan blue. The number of dead (blue) cells was counted and indicated as a percentage of all cells in the sample on the y-axis. The y-error bars represent the standard deviations of the samples. A. BHK-21 cells. B. SKBR3 cells. C. MDA-MB-231 cells. D. MCF-7 cells.

These data revealed that other breast cancer cell lines expressing different levels of the erbB receptors were also killed by the heregulin-containing viral RNAs (see manuscript for details). To determine whether expression of the erbB receptors is required for cell killing, cell lines not expressing the erbB receptors will be transfected with SVher.12 and SVher.15 viral RNA.

3) As mentioned in the previous annual report for this award, we have cloned a propagation competent virus,TE-NGR, that contains a 13 amino acid NGR (Asn-Gly-Arg)-containing peptide in place of a portion of the E2 glycoprotein receptor-binding domain. Replacement of smaller region of E2 with a smaller ligand permits production of high titer recombinant SV stocks (10^8 pfu/ml). This virus is able to replicate in BHK cells and in a human Kaposi's sarcoma cell line, SLK, that bears the CD13 receptor (Figure C). The CD13 receptor is expressed on tumor associated endothelial cells (2). Pasqualini and colleagues have demonstrated that the NGR-containing peptide binds to the CD13 receptor expressed on tumor associated vasculature

(1,5). SLK cells were infected with TE-NGR or with parent SV at a multiplicity of infection (MOI) of 5 pfu/cell to determine the ability of TE-NGR to kill SLK cells compared to parent virus (Figure D). This experiment revealed that the ability of each virus to kill SLK cells was quite low. However, TE-NGR did exhibit a tendency to kill SLK cells more efficiently than the parent virus. This experiment will be repeated at higher MOIs and in additional cell lines expressing or not expressing the CD13 receptor to further define the ability of TE-NGR to infect and kill CD13-expressing cells. Since the NGR-containing peptide preferentially binds to the "angiogenic form: of the receptor (R. Pasqualini, personal communication), the true ability of TE-NGR to infect NGR-expressing cells via a ligand-receptor interaction needs to be assayed in an in vivo tumor model.

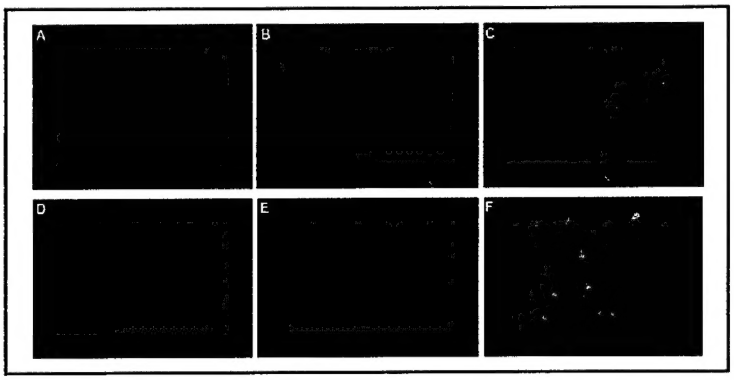

Figure C. BHK (A, B, C) and SLK (D, E, F) cells were infected with the parent virus (B, E) or with TE-NGR (C, F) at an MOI of 5 PFU/cell. The cells in panels A and D were mock-infected. After fixing with paraformaldehyde, the cells were stained with anti-SV rabbit serum and with a secondary Ab conjugated to FITC and observed with a fluorescent microscope. Numerous BHK and SLK cells stained positively (green) for the SV structural proteins when infected with the parent virus or with TE-NGR.

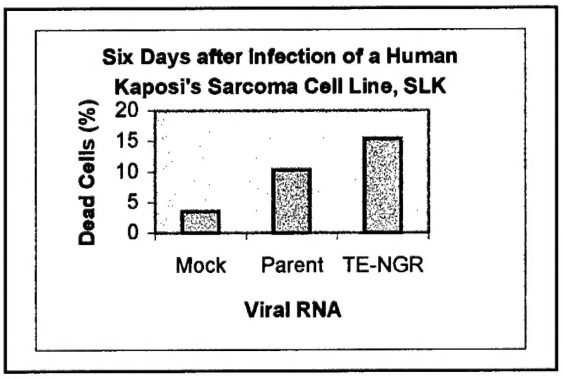

Figure D. Infection of SLK cells with parent virus and TE-NGR virus at a MOI of 5 PFU/cell. Six days after infection, the cells were harvested, stained with propidium iodide and the percentage of dead cells was determined using a FACScan flow cytometer.

Task 3 of statement of work: Test the targeted SV vectors in a mouse model of breast cancer.

We have begun to establish breast cancer tumors in Swiss athymic nude mice. Despite reports to the contrary in the literature, we were not able to establish tumors with SKBR3 cells after injection of SKBR3 cells into the mammary fat pads or into the rumps of nude mice (3).
Therefore, we have injected MDA-MB-231 cells and MDA-MB-231-HER2 cells into the mammary fat pads and into the rumps of nude mice. We were successful in establishing tumors using these cell lines and will proceed with in vivo studies using the TE-NGR virus and any other viruses that we may select in the transfection overlay assays.

III. Key Research Accomplishments.

- Isolation of TE-NGR, a propagation-competent virus containing a smaller targeting peptide ligand.
- Establishment of breast cancer tumors in nude mice.

IV. Reportable Outcomes.

- Manuscript in preparation (see appendix).

- The data obtained under this award have been used to apply for an R01 research grant from the National Institutes of Health.

V. Conclusions. We have demonstrated that it is possible to modify the receptor-binding domain of the E2 glycoprotein with a small peptide ligand and generate propagation-competent Sindbis virus. Such a virus, such as TE-NGR that is to be targeted to tumor-associated vasculature, would be able to specifically destroy tumors. The optimal location of the targeting peptide within the E2 glycoprotein remains to be determined.

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5. Pasqulini R, Koivunen E, Kain R, Lahdenranta J, Sakamoto M, Stryhn A, Ashmun RA, Shapiro LH, Arap W, Ruoslahti E. 2000. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. Cancer research. 60:722-727.

VII. Appendices.

1. Manuscript in preparation.
2. Figure 4 from manuscript.

Incorporation of the EGF-like Domain of Heregulin into the E2 Glycoprotein Modifies the Tropism of Sindbis Virus

Running Title: Modification of Sindbis Virus Tropism

Lesia K. Dropulic[1]*, J. Marie Hardwick[4], Boro Dropulic[3,5], David Irani[2,4], Holly L. Hammond[1]

[1] Department of Medicine, Division of Infectious Diseases, [2] Department of Neurology, [3] Oncology Center

The Johns Hopkins University School of Medicine

Baltimore, MD 21205

[4] Department of Molecular Microbiology and Immunology

The Johns Hopkins University Bloomberg School of Public Health

Baltimore, MD

[5] Virxsys Corporation

200 Perry Parkway

Gaithersburg. MD 20877

*Corresponding author:

Tel: 410-614-6966

Fax: 410-614-9775

e-mail: lesia@jhmi.edu

ABSTRACT

Alphaviruses are single-stranded positive-sense RNA viruses that have been developed as vectors for gene expression and are being developed as possible vaccine and gene delivery vectors for human infections and diseases. Alphaviruses are able to infect a broad range of vertebrate and invertebrate cells. To safely and efficiently prevent infections and treat diseases, a target cell-specific alphavirus vector is highly desireable. In a tumor model, using an attenuated replication-competent alphavirus vector that is targeted to receptors expressed on cancer cells may be the most efficient way of specifically killing a large number of malignant cells. To target Sindbis virus (SV), the prototype alphavirus, to receptors expressed on breast cancer cells, a putative receptor-binding domain of the SV E2 transmembrane glycoprotein was replaced with the epidermal growth factor (EGF)-like domain of the EGF-like factor, heregulin. Heregulin has affinity for the human EGF receptors, erbB-3 and erbB-4, which are expressed on certain breast cancer cell lines. After transfection of the human breast cancer cell line, SK-BR3, and the baby hamster kidney cell line, BHK-21, with the heregulin-containing SV RNA significant cytopathic effect and cell killing via apoptosis occurred only in the transfected SK-BR3 cells. The presence of the heregulin EGF-like domain in the E2 glycoprotein enabled SV to kill breast cancer cells but not BHK-21 cells. Alphaviruses that selectively kill tumor cells will enhance the potential of using alphavirus vectors to treat or prevent human diseases.

In the past decade, the development of alphaviruses, specifically SV, Semliki Forest virus, and Venezuelan equine encephalitis virus, as vectors for gene expression and as vaccine or therapeutic vectors has come to the forefront. These vectors have been proposed for use in the treatment of cancer and for vaccination against infectious diseases, such as HIV and hantavirus infections (4, 7, 18). Advantages of potential alphavirus-based therapeutic or vaccine vectors include no integration of the viral genome into the host genome, high levels of heterologous gene expression, production of high titer recombinant virus stocks, infection of proliferating and resting cells, and relatively rapid engineering of expression constructs. Alphaviruses infect a broad range of vertebrate and invertebrate cells. Hence, the ability to target alphavirus infection to specific cell types would further enhance the potential use of alphaviral vectors for therapeutic or vaccine applications.

Sindbis virus consists of a single-stranded positive sense 11,703 nucleotide RNA genome that is complexed with a capsid and surrounded by a lipid bilayer of icosahedral symmetry. The 5' two-thirds of the genome encodes the 4 nonstructural proteins and the 3' one-third encodes the structural proteins. The capsid protein, pE2 and E1 type I transmembrane glycoproteins are translated as a polyprotein from a subgenomic mRNA. During translation, the capsid protein, which possesses serine protease activity, acts in cis to release itself from the polyprotein. The glycoproteins, PE2 (precursor of mature E2) and E1, are translocated into the endoplasmic reticulum where they are folded and glycosylated. PE2 and E1 are transported as heterodimers through the secretory pathway to the cell surface where they are embedded in the plasma membrane. Prior to this, PE2 is cleaved to mature E2 in the trans-Golgi network. The mature

Sindbis virions form as the nucleocapsid buds from the plasma membrane, from which it acquires its glycoprotein spikes.

The initial step of SV infection requires binding of the virions to receptors on susceptible host cells. The putative receptor-binding domains of SV reside in the E2 glycoprotein, which consists of 423 amino acids. Studies have shown that two major neutralization epitopes in the E2 glycoprotein, spanning amino acid residues 62 to 172 and 170 to 220 residues, are particularly important for binding to cellular receptors (15, 17). These putative cellular receptor-binding domains were mapped by generating anti-idiotypic antibodies to anti-E2 antibodies that neutralized SV infection of chicken embryo fibroblasts or mouse neuronal cells. Hence, these anti-idiotypic antibodies functioned as anti-receptor antibodies that inhibited infection of cells. After binding to the cellular receptor, the virion is taken up in clathrin-coated vesicles followed by transfer to endosomes. The low pH of endosomes induces conformational changes in E1 and E2, exposing the fusion domain of E1. At this stage, homotrimers of E1 form and fuse the viral envelope with the endosomal membrane, releasing the nucleocapsid into the cytoplasm where viral replication ensues.

We have begun to explore the feasibility of using SV and SV-derived vectors as specific anti-tumor agents by attempting to modify the tropism of the virus such that it binds to receptors expressed on cancer cells. One of the main challenges of any cancer treatment is to target the treatment specifically to the cancer cells without causing adverse effects to normal cells in the body. To develop a SV that specifically binds to receptors on cancer cells and subsequently infects and kills these cancer cells, we modified the receptor-binding domain of the virus. We replaced two regions of the more N-terminal receptor-binding domain of the SV E2 glycoprotein, corresponding to amino acid residues 60 to 114 and 114 to 175, with the EGF-like domain of

heregulin. Human heregulin and the rat homologue, neu differentiation factor, have been identified as ligands for the type I receptor tyrosine kinase family members, erbB-3 and erbB-4 (2). Heterodimers of either of these receptors with erbB-2 form high affinity binding sites for heregulin. These receptors are variably expressed on epithelially derived tumors and cell lines derived from them. Here, we characterize the effects of the chimeric heregulin-containing E2 glycoprotein on SV replication and demonstrate the selective killing of human breast cancer cell lines after transfection with the heregulin-expressing SV RNAs.

MATERIALS AND METHODS

Construction of SV cDNAs encoding an heregulin-E2 glycoprotein. To have the capability to clone the EGF-like domain of heregulin into the E2 glycoprotein, three unique restriction enzyme sites, Sph I, BstE II, and Mlu I, were introduced into the SV E2 glycoprotein sequence at amino acid positions 60, 112, and 175, respectively, with little or no alterations in the amino acid sequence, by site-directed mutagenesis using a two-step polymerase chain reaction. The Sph I was introduced at E2 amino acid residue 60 by mutating nucleotide 8808 from A $\rightarrow$ T, which corresponds to a serine to cysteine amino acid change. The BstE II site was created at E2 amino acid residue 112 by mutating nucleotide 8971 from G $\rightarrow$ C, which corresponds to a serine to threonine amino acid change. The Mlu I site was created at E2 residue 174 by mutating nucleotide 9155 from T $\rightarrow$ G, which is a silent mutation. One set of mutagenic primers and one set of flanking primers were used for the construction of each unique restriction enzyme site.

The heregulin sequence was amplified from a plasmid encoding the EGF-like domain of heregulin, p-GEX-HRG$\alpha^{177-244}$ (16). The heregulin sequence was cloned in frame into the E2 sequence. Primers 5'-CTGACTGCATGCACGGTTACCAGCCATCTTGTAAAATGT-3' and 5'-GTCTGCACGCGTGGGTAACCGTCAGCACTCTCTTCTGGTA-3' were used to amplify the EGF-like domain of heregulin and replace E2 nucleotides 8810 to 8963, corresponding to the unique restriction enzyme sites, Sph I and BstE II, to create SVher.12 (figure 1). The same primer pair was used to amplify heregulin and replace E2 nucleotides 8973 to 9150, corresponding to the unique sites, BstE II and Mlu I, to create clone SVher.15. In the primer sequences, the restriction enzyme sites are underlined. Two different unique enzyme sites were designed into each primer so that the same primers could be used for amplification and subcloning into each region. Replacement of E2 nucleotides 8810 to 8963 (SVher.12) resulted in a chimeric E2 sequence that was 59 basepairs (bp) or approximately 2200 daltons larger than the wild-type E2 sequence. Replacement of E2 nucleotides 8973 to 9150 (SVher.15) resulted in a chimeric E2 sequence that was 36 bp or 1430 daltons larger than wild-type E2. The heregulin-containing clones were sequenced using the dideoxy-mediated chain termination reaction to ensure correct plasmid construction.

Production of viral RNA. The SVher.12 and SVher.15 plasmids were linearized with Sac I for template production. Viral RNA was transcribed in vitro using 0.6 μg of template, SP6 DNA-dependent RNA polymerase (Life Technologies, Inc.), 1.0 mM each of rATP, rCTP, rUTP, 0.8 mM rGTP, and 1mM cap analog (7^{m}G5'ppp5'G) in a 20 μl reaction. The reaction was incubated for 4 hours at 37 ° C. The quality and quantity of synthesized viral RNA was determined by agarose gel electrophoresis. An aliquot of RNA was diluted 1:5 in RNA loading buffer (Sigma) containing formaldehyde, bromphenol blue, and ethidium bromide, heated at 65 °

C for 10 minutes, and loaded onto a 0.8 % agarose gel. The concentration of RNA was determined by comparison to a known RNA standard (Life Technologies, Inc.). The gel was viewed using the Alpha Innotech Imager 2200, and the RNA concentration was determined by comparison to a known RNA standard (Life Technologies, Inc.) using spot densitometry.

Transfection of mammalian cell lines with viral RNA. Cells were seeded 24 hours prior to the time of transfection in order to achieve 80 % confluency. RNA-lipid complexes were formed in 24 well plates. Three hundred microliters of serum-free medium, Opti-MEM (Life Technologies, Inc.), were added to each well with 2.5 μg of Lipofectin (Life Technologies, Inc.) for BHK-21 transfection or 5.0 μg of DMRIE-C (Life Technologies, Inc.) for SK-BR3 transfection. The medium and lipids were incubated at room temperature for 30 minutes. Then, 2.5 μg of SV RNA was added to each well containing Opti-MEM and lipid. The cell monolayers were washed once with Opti-MEM, and the lipid/RNA complexes were immediately added to the cells and incubated for 4 hours at 37 ° C in a CO_2 incubator. At the end of the incubation, the RNA/lipid complex-containing medium was replaced with complete growth medium containing 2 % fetal calf serum (FCS). The cells were incubated overnight in a 37 ° C CO_2 incubator. Twenty-four or forty-eight hours after transfection, the cells were assayed via immunofluorescence or for trypan blue or propidium iodide exclusion.

Radioimmunoprecipitation of cell lysates derived from breast cancer cells transfected with viral RNA. In vitro transcribed heregulin-encoding SV RNA was transfected into BHK-21 cells. At 20 hours after transfection, the cells were starved for one hour in methionine- and cysteine-free modified Eagle medium and labeled with 100 μCi/ml of TranS^{35}label for 2 hours at 37 ° C. After completion of radiolabelling, the cells were placed on ice, washed three times with cold 1 X PBS, and lysed with radioimmunoprecipitation assay buffer (150 mM NaCl, 10 mM

EDTA, 1 % Triton X-100, 0.5 % deoxycholate, 0.1 % sodium dodecyl sulfate, 50 mM Tris Cl [pH 8.0]). For immunoprecipitation, anti-SV rabbit serum (1: 80 dilution) or anti-E2 monoclonal antibodies # 2 and # 136 (1: 100 dilution) were added to 10^6 counts per minute of cell lysate and incubated overnight at 4 ° C. Subsequently, protein A sepharose beads (Pharmacia) were added at a 1: 5 diltuion for the precipitation of immune complexes overnight at 4 ° C. The immune complexes were washed 3 times with buffer A (0.01 M Tris Cl [pH 7.5], 0.15 M NaCl, 0.002 M EDTA, 0.2 % Nonidet P-40), two times with buffer B (0.01 M Tris Cl [pH 7.5], 0.50 M NaCl, 0.002 M EDTA, 0.2 % Nonidet P-40), and one time with buffer C (0.01 M Tris Cl [pH 7.5]). The immune complexes were resuspended in sample buffer containing sodium dodecyl sulafate and β-mercaptoethanol and boiled for 5 min. The immunoprecipitated proteins were separated by electrophoresis through an SDS-15 % polyacrylamide gel. The gel was processed for autoradiography.

Immunofluorescence assay. The assay was performed on cells in 4 well chamber slides or on cells that were harvested onto slides from post-transfection supernatant fluids using a cytospin. The cells were fixed with 4 % paraformaldehyde with or without 0.1 % Triton X-100. After fixation, the cells were washed 4 times with 1 X PBS. Primary antibody, anti-SV rabbit serum or anti-heregulin goat serum (Santa Cruz), in 1 X PBS with 2% FCS, was added to the cells and incubated for 20 minutes at 37 ° C. The cells were washed 3 times with 1X PBS and the secondary antibody (Alexa Fluor 488 goat anti-rabbit, Alexa Fluor 594 rabbit anti-goat, Molecular Probes) in 1 X PBS with 2% FCS was added. The cells were incubated for 20 minutes at 37 ° C. The cells were washed 3 times with 1X PBS and rinsed in distilled water. Coverslips were mounted with Permafluor. The cells were viewed with a Zeiss fluorescent microscope.

Determination of cell death. To determine the number of dead cells present in transfected cultures 24 or 48 hours after transfection, the cells were stained with Trypan blue and counted under a light microscope. Alternatively, the cells were stained with propidium iodide, and the number of dead cells were counted using a flow cytometer. To detect apoptosis-induced nuclear DNA fragmentation, the cells were transfected with the appropriate viral RNAs and then the ApoAlert DNA Fragmentation Assay Kit (Clontech) was used to detect apoptosis. This assay is based on the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end-labeling (TUNEL) reaction. TdT catalyzes the incorporation of fluorescein-dUTP at the 3'-hydroxyl ends of fragmented DNA. The fluorescein-labeled DNA was detected via fluorescence microscopy.

RESULTS

Analysis of Sindbis virus structural protein synthesis. In order to target SV to the erbB receptors, sequences encoding the putative receptor-binding domain of the SV E2 glycoprotein, spanning amino acid residues 61 to 173, were replaced with the sequence for the EGF-like domain of heregulin (figure 1). Clone SVher.12 contains the heregulin sequence in place of E2 amino acid residues 61 to 111. Clone SVher.15 contains the heregulin sequence in place of E2 amino acid residues 115 to 173. First, we determined what effect the heregulin sequence had on viral structural protein synthesis and on viral replication. Because alphaviruses contain a positive, message-sense, RNA genome, infectious RNA transcripts can be produced in vitro from a plasmid containing the SV genome cloned behind a bacteriophage promoter. To determine whether the heregulin-E2 glycoprotein was synthesized, BHK-21 cells were transfected with SV genomic RNA derived from the cDNA clones, SVher.12 and SVher.15. Cell lysates derived from the transfected cell cultures were immunoblotted with an anti-α-heregulin antibody that

recognizes an epitope present in the EGF-like domain of heregulin. Synthesis of the chimeric heregulin-E2 glycoprotein was detected at the expected molecular weight in BHK-21 cells transfected with SVher.12 and SVher.15 RNAs (figure 2A). The size of the heregulin-containing E2 approximated the size of wild-type E2. In contrast, no heregulin-containing E2 was detected in mock-transfected or parent virus RNA-transfected BHK-21 cells. However, the anti-α-heregulin antibody detected endogenous heregulin in MDA-MB-231 cells.

To determine whether the presence of the EGF-like domain of heregulin in E2 affected the synthesis and processing of the E1 and E2 glycoproteins, we performed an immunoprecipitation assay using radiolabelled cell lysates derived from BHK-21 cells transfected with SVher.12 and SVher.15 RNAs. Immunoprecipitation of these cell lysates with anti-SV rabbit serum and with two monoclonal antibodies (#2 and # 136), that recognize different epitopes of E2, revealed that the heregulin-containing precursor of E2, PE2, was processed to the mature chimeric E2 glycoprotein (figure 2B). The chimeric PE2 and mature E2 were readily detected after immunoprecipitation with anti-SV rabbit serum and with anti-E2 monoclonal antibody #2. Unlike monoclonal antibody #2, monoclonal antibody # 136 co-immunoprecipitates E2 with E1 (figure 2B, lower panel). In SVher.12 and SVher.15-transfected cultures, monoclonal antibody #136 predominantly immunoprecipitated the heregulin-containing E2. E1 was barely detectable. These data suggest that the usual heterodimeric interaction between the E1 and E2 glycoproteins was disrupted because of the presence of the EGF-like domain of heregulin in E2. However, despite this alteration in the heterodimerization of E1 and E2, the E2 precursor was processed into the mature chimeric E2 glycoprotein. The amount of E1 and chimeric E2 glycoproteins synthesized in the cell cultures transfected with the heregulin-containing viral RNAs was significantly less compared to the amount of glycoproteins synthesized in cell cultures

transfected with the same amount of parent SV RNA (figure 2B). These results indicate that the replication of the heregulin-containing SV clones was attenuated because of the substitution of regions of the putative E2 receptor-binding domain with the EGF-like domain of heregulin.

To determine whether the heregulin-containing E2 glycoprotein was transported to the cell membrane and expressed on the surface of the cell, an immunofluorescence assay was performed using the anti-SV rabbit serum and the anti-heregulin antibody. Flourescent staining with these antibodies was detected in the cytoplasm (data not shown) and on the surface of BHK-21 and SK-BR3 cells that had been transfected with SVher.12 and SVher.15 RNAs (figure 3). This assay confirmed that the structural proteins, including the heregulin-containing E2 glycoprotein, are synthesized in transfected SK-BR3 and BHK-21 cells and that the heregulin-containing E2 is expressed on the surface of these cells. These results suggest that the modified E2 glycoprotein traffics through the secretory pathway to the surface of the cell where it is inserted into the plasma membrane. Since SV derives its envelope from the plasma membrane of the cell, the heregulin-containing E2 glycoprotein would be expected to be incorporated into the envelope of the virion.

We attempted to more definitively identify whether the E1 and chimeric E2 glycoproteins were expressed on the surface of the virion. BHK-21 cells were electroporated with either SVher.12 or SVher.15 RNA. Transfection efficiencies of up to 90% were achieved with electroporation. At 20 hours after electroporation, the cells were metabolically labeled with Tran^{35}Slabel for 3 hours. The supernatant fluids were harvested and clarified of cellular debris. Virions in the supernatant fluids were pelleted at 26,000 rpm for 2 hours at 4 ° C in a Beckman ultracentrifuge and analyzed on a polyacrylamide-SDS gel. Autoradiography was performed. These experiments revealed that a very small amount of virus was produced, suggesting that the

heregulin-sequence most likely altered virus particle assembly and release (figure 2C). The pattern of proteins detected in the SVher.12 virions mimicked the pattern seen in cell lysates immunoprecipitated with anti-SV rabbit serum and with monoclonal antibody #2. The heregulin-containing PE2 was incorporated into the SVher.12 virions along with the mature heregulin-E2, suggesting that the precursor was not completely processed into the mature protein. A very small amount of the E1 glycoprotein was detected. The killing of breast cancer cells after transfection with heregulin-containing viral RNAs at a frequency greater than the transfection efficiency (see figure 5 below) suggests that the heregulin-containing viruses may spread through the culture, in the absence of significant levels of extracellular virus, via direct cell-to-cell transmission.

Replication of the E2-heregulin-containing viruses in mammalian cell lines. Despite the effects of the heregulin sequence on structural protein synthesis and virus production, we proceeded to determine whether transfection of the heregulin-containing SV RNA transcripts could induce cell death in human breast cancer cells expressing the appropriate erbB receptors. BHK-21 cells and SK-BR3 human breast cancer cells were transfected with 2.5 μg of RNA using either Lipofectin or DMRIE-C, respectively. The transfection efficiency determined using an SV replicon expressing lacZ was 13 % in BHK-21 cells and 10 % in SK-BR3 cells. Twenty-four and forty-eight hours after transfection, the cells were observed for a cytopathic effect. We detected a significant cytopathic effect only in the human breast cancer cells that were transfected with SVher.12 or SVher.15 viral RNA (figure 4G). Repeatedly, no cytopathic effect was observed in BHK-21 cells transfected with the modified viruses (figure 4C).

The number of dead cells in the transfected cultures was quantified using the Trypan blue or propidium iodide exclusion assay (figures 5). The parent virus RNA killed both cell lines, but

was less efficient in killing SK-BR3 cells. At 48 hours after transfection, 88 ± 2.0 % of BHK-21 cells were killed by the parent virus RNA versus 20 ± 0.7 % of the SK-BR3 cells (figures 5A and 5B). Transfection of SK-BR3 cells with SVher.12 RNA killed 26 ± 1.0 % of the cells, and transfection with SVher.15 RNA killed 18 ± 0.8 % of the cells (figure 5B). Mock-transfected and SV replicon-transfected SK-BR3 cells exhibited 6 ± 1.1 % and 7 ± 1.4 % dead cells, respectively. Transfection of BHK-21 cells with SVher.12 RNA or SVher.15 RNA did not result in any significant killing of BHK-21 cells compared to mock-transfected or SV replicon-transfected BHK-21 cell cultures (figure 5A). Transfection of SK-BR3 cells with SVher.12 RNA resulted in enhanced killing of SK-BR3 cells compared to transfection with parent SV RNA, 26 ± 1.0 % dead cells versus 20 ± 0.7 % dead cells, respectively (figure 5B). These data demonstrate that we successfully modified the tropism of SV, such that transfection with the heregulin-containing viral RNAs induced death in the SK-BR3 human breast cancer cell line, but not in the BHK-21 cell line.

Other breast cancer cell lines, which express different levels of the erbB receptors, were also killed by the heregulin-containing viral RNAs. These included MDA-MB-231 cells, which, relative to the SK-BR3 cells, express low levels of erbB-2 and erbB-3 and MCF-7 cells which express low levels of erbB-2, but high levels of erbB-3 (1). SK-BR3 cells express very high levels of erbB-2 and levels of erbB-3 that are between those expressed in MDA-MB-231 and MCF-7 cells. The degree of cell death 48 hours after transfection of MDA-MB-231 cells with parent virus RNA was 23 ± 1.0 % (figure 5 C). After transfection of MD-MB-231 cells with SVher.12 RNA the degree of cell death was 27 ± 1.8 % (figure 5 C). The degree of cell death after transfection of MCF-7 cells with parent virus RNA was 20 ± 0.2 % and 17 ± 0.6 % after transfection with SVher.12 RNA (figure 5 D). These results suggest that the ability of the

heregulin-containing viral RNAs to kill breast cancer cells is not related to the level of expression of the erbB receptors on different breast cancer cell lines.

We proceeded to begin to investigate the mechanism of killing of breast cancer cells by the heregulin-containing SV RNAs. We noted that SK-BR3 cells transfected with SVher.12 or SVher.15 RNA exhibited extensive membrane blebbing, a characteristic of apoptosis (figure 4G). Given this feature and the fact that SV infection is known to kill cells via apoptosis (11), the transfected SK-BR3 cell cultures were assayed for DNA fragmentation, an hallmark of apoptosis, using the TUNEL reaction (figure 6). Dying cells in the supernatant fluids were harvested and deposited on slides using a cytospin. The SK-BR3 cultures transfected with SVher.12 and SVher.15 RNAs revealed numerous apoptotic cells compared to an occasional apoptotic cell in mock-transfected cultures (figure 6A, B, and C). SK-BR3 cells transfected with parent virus RNA were also apoptotic (figure 6D). To prove that the apoptotic cells were the cells that were transfected with the heregulin-containing SV RNAs, SK-BR3 cells were transfected with an heregulin-containing SV vector expressing the enhanced green fluorescent protein (EGFP), SVher.12EGFP. These results demonstrated that the cells that expressed EGFP were the cells that were dying an apoptotic death (figure 6 E and F).

DISCUSSION

The ability to target viral vectors to specific cell types is an important goal in vector development for therapeutic or vaccine applications. In this study, we demonstrate that the tropism of SV was modified by replacing regions of the putative receptor-binding domain of the E2 glycoprotein with the EGF-like domain of heregulin. The SV transcripts expressing the heregulin-containing E2 glycoprotein were able to induce cell death of human breast cancer cells

but not of BHK-21 cells, which are highly sensitive to the cytolytic effects of SV. This ability of SV to preferentially kill breast cancer cells enhances the potential use of alphaviruses as therapeutic vectors.

Our goal was to modify the tropism of SV by replacing the putative receptor-binding domain in E2, that has been proven to be important in binding to mammalian cells (15), with a ligand that would re-direct the binding of SV to the erbB receptors expressed on breast cancer cells. Our analysis of structural protein synthesis revealed that the chimeric heregulin-E2 glycoprotein was synthesized. However, the presence of the EGF-like domain of heregulin altered the heterodimerization of E1 and E2, and as a result, hindered the assembly and release of virions, resulting in a significant decrease in virus production. Hence, the cell killing experiments were performed by transfecting cells with SV RNA transcripts. In the setting of similar transfection efficiences, but generally greater in BHK-21 cells, cytopathic effect after transfection was observed only in the human breast cancer cell lines. With transfection, the binding and entry steps of the virus life cycle are bypassed and virus yield depends only on RNA amplification and virion assembly and release from transfected cells. Our inability to passage the heregulin-containing virions after transfection suggests that the presence of the heregulin sequence within the E2 sequence affects one or more of these steps resulting in a significant decrease in virus production.

Despite the decrease in virus production, a dramatic difference in the ability of the transfected SV RNA transcripts to selectively kill breast cancer cells was demonstrated. The preferential killing of breast cancer cells cannot be readily explained by the binding and entry of the modified virions into breast cancer cells via a ligand-receptor, i.e. via an heregulin-erbB-2/erbB-3, interaction. With transfection, binding and entry are bypassed. Hence, we considered other

mechanisms that may be playing a role in the selective killing of breast cancer cells by the SV heregulin-containing RNAs. The region spanning E2 amino acids 62 to 172 has been demonstrated to be important for SV binding to BHK-21 cells (3, 10). By replacing this region with the EGF-like domain of heregulin, we may have destroyed the ability of the virus to spread in BHK-21 cells; hence, no cytopathic effect was observed in this cell line. However, possibly, the small amount of heregulin-containing virus that is produced may retain the ability to bind to, enter, and kill neighboring breast cancer cells. Perhaps, with our assays, we were unable to detect that this is occurring through a heregulin/erbB-2, -3 receptor interaction, since competition experiments with a GST-heregulin fusion protein and with an anti-HER-3 receptor-blocking antibody did not alter the ability of the SV-heregulin transcripts to selectively kill SK-BR3 cells (data not shown). The ability of the heregulin-containing virions to spread in breast cancer cells is supported by the fact that the amount of cell death in transfected breast cancer cells is significantly greater than the transfection efficiency.

The challenge of modifying the envelope of SV has been undertaken by other investigators. Heterologous sequences have been engineered into the SV structural genome to express antigens on the surface of the Sindbis virion or to target SV to specific cells. London and colleagues used random insertion mutagenesis to identify sites in the SV genome permissive for insertion of an 11-amino acid protective epitope from the Rift Valley fever virus (12). Insertion of this neutralizing epitope at two different sites, after residue 3 and after residue 244, in E2, allowed recovery of chimeric viruses with growth properties similar to the parental virus. The epitope was expressed on the virion surface. These studies proved that it is possible to modify the E2 glycoprotein with a heterologous sequence and still produce infectious virions. The 11 amino acid Rift Valley fever virus epitope is much smaller than the 68 amino acid sequence we

engineered into SV. Additional studies using the random insertion mutagenesis strategy revealed that insertions in the region between E2 amino acids 69 and 74 allow normal particle assembly and release, but block virus entry at the level of binding (5). Ohno et al. used this information to develop a non-replication competent targeted SV particle by inserting the IgG-binding domain of protein A between E2 amino acids 69 to 74 (13). Incubation of such modified SV particles with monoclonal antibodies against specific cell-surface receptors enabled infection of cell lines that are not readily infected by SV.

Single amino acid changes in E1 and E2 can result in dramatic changes in the biological properties or cell tropism of alphaviruses (14). Recently, Gardner et al. serially propagated SV in highly enriched precursors of a human myeloid dendritic cell subpopulation to derive a variant that could target the human dendritic cell (6). A single amino acid substitution at E2 position 160 conferred SV with the ability to efficiently infect dendritic cells. However, the dendritic cell tropic and non-dendritic cell tropic SV equivalently infected BHK-21 cells. Gardner et al. stressed the importance of using a minimally passaged virus and a relevant cell type, such as a primary non-transformed human cell line, to derive alphavirus variants that infect specific cell types. Infection of murine dendritic cells did not translate to efficient human dendritic cell infection. Using a similar strategy, serial passaging of the supernatant fluids derived from human breast cancer cells transfected with the heregulin-containing SV RNAs may select for a virus with a mutation (s) that enables more efficient infection and replication of the modified viruses in human breast cancer cells (8). This will enable us to study the modified virions in greater detail and to determine whether the EGF-like domain of heregulin is definitively expressed on the surface of the virion. The presence of the 204 bp heterologous sequence in the SV genome makes reversion to a wild-type SV sequence highly unlikely.

The use of viruses a tumoricidal agents has gained momentum in recent years. Some viruses, such as reovirus and Newcastle disease virus exhibit inherent tumor-selectivity (9). Other viruses, such as the adenovirus, ONYX-015, and the herpes simplex virus, G207, have been genetically modified to enable specific replication in cancer cells. Hence, there is precedence with other viruses for enabling them to specifically replicate in and destroy tumor cells. The ability to target SV infection to specific cancer cells would expand the usefulness of engineered alphaviruses as tumoricidal agents. Possibly by identifying appropriate ligands that are smaller in size, which have been proven by other investigators to be less likely to disrupt important functions of the E1 and E2 glycoproteins (12), we will be able to create Sindbis viruses that are exclusively targeted to receptors expressed on cancer cells.

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FIGURES

Figure 1. Schematic diagrams of the genome of the heregulin-containing Sindbis virus clones. SV E2 amino acids 61 to 111 and 115 to 173 were replaced with the EGF-like domain of heregulin to create SV clones SVher.12 and SVher.15, respectively. The unique restriction enzyme sites for replacing these two regions of the E2 receptor-binding domain are depicted. The parent genome contains the unique restriction enzyme sites with no other modifications from the wild-type SV sequence.

Figure 2. A. Synthesis of the heregulin-containing E2 envelope glycoprotein in BHK-21 cells. Cell lysates of BHK-21 cells transfected with parent, SVher.12 or SV her.15 RNA were immunoblotted with a polyclonal antibody that recognizes an epitope in the EGF-like domain of α-heregulin. The anti-heregulin antibody detected the heregulin-containing E2 glycoprotein (arrow) in BHK-21 cells transfected with SVher.12 and SVher.15 RNA (lanes marked 12 and 15, respectively). Lane C: cell lysate from the human breast cancer cell line, MDA-MB-231, which serves as a positive control for the antibody. The 30 kDa band is a mature form of heregulin that contains the EGF-like domain (arrowhead). The 44 kDa glycosylated heregulin protein is detected on longer exposure of the blot. Lane M: mock-transfected BHK-21 cells. Lane P: BHK-21 cells transfected with parent virus RNA. The higher molecular weight band is a non-specific band recognized by the heregulin antibody in BHK-21 cells.

B. Analysis of viral structural protein synthesis. BHK-21 cells were transfected with yeast RNA parent virus RNA, or heregulin-containing viral RNA. Cell lysates were immunoprecipitated

with anti-SV rabbit serum or with the anti-E2 monoclonal antibodies #2 and #136. M = mock, P = parent virus RNA, 12 = SVher.12 virus RNA, and 15 = SVher.15 virus RNA. The gel was overexposed, as demonstrated by the lanes containing lysates from cells transfected with parent virus RNA, in order to be able to visualize the bands derived from the SVher.12- and SVher.15-transfected cells. The migration of molecular weight markers is indicated on the left side of the gel. PE2, precursor of E2. E2, mature E2 glycoprotein. C, capsid protein.

C. Pelleting of radiolabelled heregulin-containing virions. Virions were pelleted from post-electroporation supernatant fluids and analyzed on a 12 % polyacrylamide-SDS gel. Lane M: supernatant fluids derived from mock-transfected BHK-21 cells. Lane P: supernatant fluids derived from parent virus RNA-transfected BHK-21 cells. Lane 12: supernatant fluids derived from SVher.12-transfected BHK-21 cells. The broad band migrating at approximately 50 kilodaltons in lane P represents the E1 and E2 glycoproteins which are of similar molecular weight and charge and hence, migrate a similar distance on the gel. C, capsid protein.

Figure 3. Immunofluorescence assay of BHK-21 and SK-BR3 cells. BHK-21 (A-D) and SK-BR3 (E-H) cells were transfected with SVher.15 RNA (A, C, E, G) or yeast RNA (B, D, F, H), fixed with 4 % paraformaldehyde, and then stained with anti-SV rabbit serum (A, B, E, F) or with anti-heregulin antibody (C, D, G, H). Similar results were obtained after transfection with SVher.12 RNA, however, the immunofluorescence staining was less intense.

Figure 4. Cytopathic effect in transfected BHK-21 and SK-BR3 cell lines. BHK-21 and SK-BR3 cells were transfected with SVher.15 viral RNA. Forty-eight hours after transfection, the cells were visualized via light microscopy. Figures A, B, C, and D are BHK-21 cells transfected

with yeast, parent , SVher.15 or psinlacz replicon RNAs, respectively. Figures E, F, G and H are SK-BR3 cells transfected with yeast, parent, SVher.15 or psinlacZ replicon RNAs, respectively. Note membrane blebbing in SK-BR3 cells transfected with SVher.15 RNA. Similar results were obtained when the cells were transfected with SVher.12 RNA. (200X magnification).

Figure 5. Quantification of cell death in transfected mammalian cell lines. Forty-eight hours after transfection, the cells were harvested and stained with Trypan blue. The number of dead (blue) cells was counted and indicated as a percentage of all cells in the sample on the y-axis. The y-error bars represent the standard deviations of the samples. A. BHK-21 cells. B. SK-BR3 cells. C. MDA-MB-231 cells. D. MCF-7 cells.

Figure 6. Detection of apoptosis. Forty-eight hours after transfection of SK-BR3 cells with A. SVher.15 RNA, B. yeast RNA, C. SVher.12 RNA or D. parent virus RNA, dying cells in the supernatant fluids were harvested and deposted on slides using a cytospin. Apoptosis-induced nuclear DNA fragmentation was detected via TUNEL and fluorescence microscopy. The green and greenish-yellow appearing cells are positive for the TUNEL reaction. E and F. SK-BR3 cells were also transfected with an heregulin-containing SV vector expressing EGFP, dsSVher.12EGFP. E. Phase contrast microscopy of transfected cells. F. Fluorescent microscopy of transfected cells.

Figure 4